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The Immunosuppressive Effect of Mycoplasma Infection

I. EFFECT ON THE HUMORAL AND CELLULAR RESPONSE

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Summary. Mycoplasma-induced immunosuppression in rats was demonstrated with *Mycoplasma arthritidis* strain PN. Immunosuppression involving the humoral antibody response was complete or partial when mycoplasma was injected concurrently with the antigen. Mycoplasma did not interfere with the antibody response when injected after primary immunization.

Inhibition of blast transformation was shown to occur in lymphocytes derived from rats infected with the above mycoplasma. The inhibition was partial or complete depending on the interval between the infection and the time when the lymphocytes were taken from the animal, and blast transformation reverted to normal when the infection was over.

Although the question of mechanism remains open, it was concluded that immunosuppression might be the result of the effect of *M. arthritidis* on a particular population of cells.

INTRODUCTION

Preliminary experiments have shown that *M. arthritidis* and *M. pneumoniae* can suppress the serum antibody response of laboratory animals (Kaklamanis, Thomas, Hsiung, Siskind and Stavropoulos, 1966, unpublished data). It has also been shown that several species of mycoplasma inhibit lymphocyte transformation induced *in vitro* by PHA (Copperman and Morton, 1966), or specific antigens (Simbercoff, Thorbecke and Thomas, 1969).

The following experimental work was undertaken to study further the effect of *M. arthritidis* on the immune response and to investigate the mechanism of immunosuppression. For this purpose two series of experiments were set up: (a) Titration of anti-bacteriophage ϕ 5 antibodies and (b) Investigation of PHA-induced transformation of lymphocytes from infected and non-infected animals.

Mycoplasma arthritidis was chosen as it is easy to grow and highly pathogenic for the rat. The infected rats developed an acute arthritis easy to detect clinically. Bacteriophage ϕ 5 was considered a suitable antigen as the method for testing neutralizing antibodies is very sensitive and specific.

Our preliminary results were presented at the autumn meeting of the British Society for Immunology (Kaklamanis and Pavlatos, 1969).

MATERIAL AND METHODS

Mycoplasma arthritidis strain PN was kindly supplied by Dr L. Thomas of Yale Univer-

sity. This strain had been isolated from a spontaneous abscess of the neck of a rat by Dr Ward of Utah University. The 15th subculture in broth, which was shown to be pathogenic for the rat, was divided into 1 ml aliquots and kept at −20°. This was used as the inoculum for all experiments.

The mycoplasma was grown in broth consisting of seven parts Difco PPLO broth, two parts unheated horse serum (B-D Meieux) and one part 25 per cent freshly prepared yeast extract. The medium was supplemented with 1 per cent arginine and 1000 μg penicillin/ml. The pH was adjusted to 7. An 18-hour culture usually had a count of $2–5 \times 10^9$ micro-organisms/ml.

Animals

Sprague-Dawley rats, bred in our animal house and weighing 200–250 g, were used for all experiments.

Immunization

Bacteriophage ϕ 5 was used as antigen. It is a Pseudomonas phage isolated from sewage (Pavlatou and Kaklamanis, 1961). The bacteriophage was propagated in nutrient broth (Difco) on the indicator strain, *Pseudomonas pyocyanea* 30.

Test and control animals were immunized by intraperitoneal (i.p.) inoculation of 1 ml bacteriophage suspension containing 5×10^9 PFU (plaque-forming units). A booster dose of 0·1 ml of the same lysate was given by the same route 2 weeks after the first injection.

Infection

The test animals were inoculated with 2 ml of an 18-hour culture of *M. arthritidis* strain PN containing 1×10^{10} CFU (colony-forming units) either concurrently or 10 days after primary immunization. Control animals were also injected with 2 ml/PPLO broth (the medium used for growing the mycoplasma).

Cell culture

Donor rats were killed by intravenous injection of air at various times after immunization. The submaxillary and mesenteric lymph nodes were excised aseptically trimmed and washed in Eagle's MEM medium for suspension cultures (Difco). The lymph nodes were then minced with scissors and the cells gently suspended in the above medium supplemented with 0·6 per cent glutamine.

These cell suspensions were filtered through two layers of sterile gauze and washed three times with the same medium. They were then adjusted to 2×10^6 cells/ml in the same medium supplemented with 20 per cent inactivated calf serum (Difco). The suspension was distributed in 4-ml aliquots in glass universal containers of 25 ml capacity. The lymph nodes of each animal were processed and cultured separately. Four cultures were prepared from each animal and to two, 0·1 ml of PHA M. (Difco) was added (each vial was reconstituted in 5 ml Eagle's medium). The containers were incubated at 36° for 96 hours. Three hours before harvest 10 μg of colcemid (CIBA) was added and the tubes were reincubated at 36°. At the end of this period, the cell suspension was spun down at 800 rev/min × 10 minutes and the supernatant was removed. The cells were resuspended in 5 ml of 1 per cent sodium citrate prewarmed to 36° and incubated at 36° for 30 minutes, after which they were spun down at 800 rev/min × 10 minutes, resuspended in 5 ml freshly

prepared fixative [3 : 1 absolute methanol : glacial acetic acid] and allowed to stand at 4° for 30 minutes. The cells were again sedimented at 800 rev/min ×10 minutes and the supernatant poured off and this procedure was repeated till the supernatant fixative was clear.

The sedimented cells were suspended in a few drops of fixative, spread on clean coverslips, air dried and stained with Giemsa.

The examination of the slides for the proportion of transformed cells was done by the two of us independently and the recorded results express the average of the two readings.

Assay for phage neutralization by antisera

Animals were bled from the tail vein at various time intervals following primary and secondary immunization with bacteriophage and their sera were kept individually at −20° till they were assayed for phage-neutralizing antibodies.

Inactivation of PFU was measured using the double agar layer technique described by Adams (1959). The phage was diluted in nutrient broth (Difco) to give a lysate of 5×10^2 PFU/ml. The antisera were diluted two-fold serially in nutrient broth. The inactivation of phage by antibody follows first order kinetics and the constant, which is considered to be a measure of neutralizing antibody, was calculated using the relationship

$$K = \frac{2{,}3\ D}{t} \log_{10}\left(\frac{Po}{P}\right),$$

where D is the reciprocal of the serum dilution, Po is the phage assay at zero time, P is the phage assay at time t minutes.

RESULTS

PATHOGENICITY

Arthritis usually appeared in the majority of rats, 3–4 days after injection. The arthritis was progressive involving various joints of the limbs. Multiple joint involvement was the rule and occasionally it extended to the interspinal articulations. Histologically, the arthritis was characterized by suppuration, marked distension of the joint space, oedema and increased periarticular ground-substance. The injected mycoplasma could always be isolated from the arthritic joints. Some rats developed urethritis, rhinitis and conjunctivitis, from which the injected mycoplasma could always be isolated, and/or knobby swelling of the tail.

Blood cultures for mycoplasma from rats with arthritis were positive 40 days after injection.

Cultures of different organs were also positive for several days. Cultures of spleen tissue (1 : 200 w/v PPLO-Broth) were positive for 30 days, while cultures of synovium were generally positive for longer periods but only in rare cases were still positive for 50 days after injections of *M. arthritidis*.

ANTIBODY RESPONSE OF IMMUNIZED AND CONCURRENTLY INFECTED ANIMALS

Sera from all animals were tested at 10-day intervals after primary immunization with phage. Fig. 1 shows the results of the neutralizing assay of one experiment. All control immunized rats responded to phage ϕ 5 from the first 10 days after primary immuniza-

tion, developing a higher titre of antibodies after the booster injection. In all infected animals suppression of immune response was demonstrated. After the primary injection of bacteriophage most of these animals did not develop any antibody response. After the booster injection 2/5 animals did not react at all to the antigenic stimulus, while the rest developed a poor antibody response.

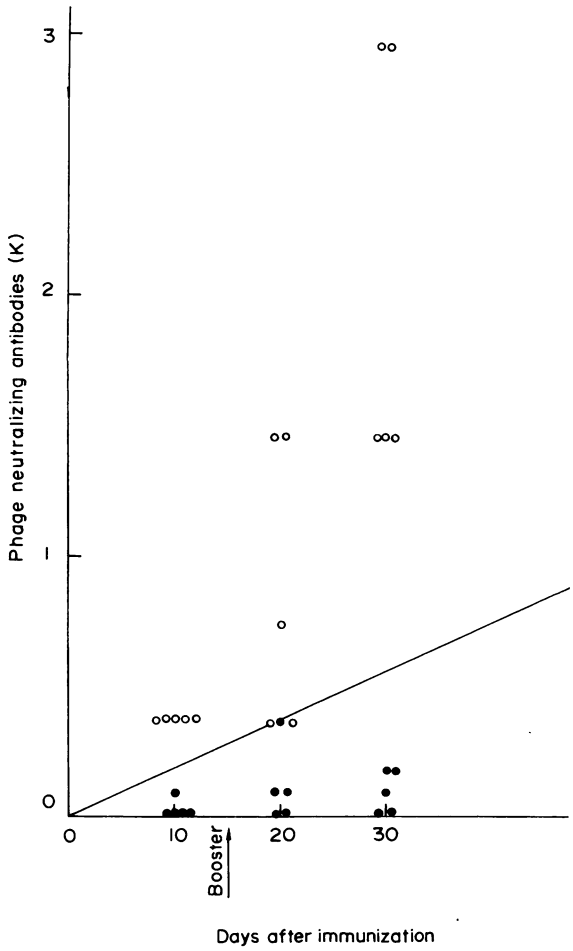

FIG. 1. Neutralizing antibodies to bacteriophage ϕ 5. ○, Indicates non-infected immunized animals; ●, indicates animals immunized and concurrently infected (representative experiment).

ANTIBODY RESPONSE OF ANIMALS INFECTED AFTER IMMUNIZATION

Fig. 2 shows the effect of mycoplasma infection 10 days after primary immunization. The same figure includes controls (non-infected rats) and a group of animals infected at the time of immunization. Suppression of antibody response is demonstrated only in the latter group of animals. Rats infected 10 days after the primary immunization responded to the antigenic stimulus like those of the control group.

All infected animals in both series of experiments had arthritis involving one or more

limbs, but their general condition was good with no loss of weight or significant reduction of activity.

Lymphocyte blast transformation

Cells from control animals, injected with PPLO broth, remained small with dense nuclei when no PHA was added. The addition of PHA resulted in transformation of the lymphocytes into immature 'blast' cells and the appearance of many mitotic figures.

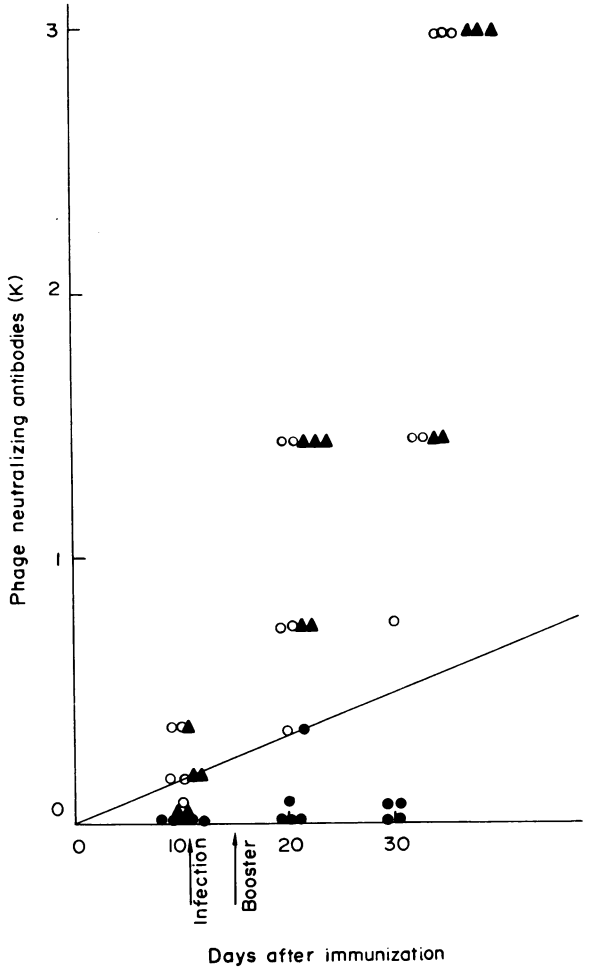

FIG. 2. Neutralizing antibodies to bacteriophage ϕ 5. ○, Indicates non-infected immunized animals; ●, indicates immunized and concurrently infected animals; ▲, indicates animals infected 10 days after primary immunization (representative experiment).

In contrast, the addition of PHA did not result in lymphocytic blastoid conversion of cells from arthritic rats. The inhibition of the mitotic effect of PHA was partial or complete depending on the time interval between the inoculation and the culture. As can be seen from Fig. 3, 10 days after the inoculation the inhibition of blast transformation was obvious and between days 20–30 it was almost complete. Thirty-five days after the inoculation,

transformation was about 50 per cent of the control transformation and by the 50th day it had reverted to normal. At that time in the animals of this group the arthritis had subsided completely and mycoplasma was no longer isolated from the involved joints. Transformation in the controls varied from 50–70 per cent with a mean of 60 per cent (Fig. 3).

It must be emphasized that cultures of the lymphocyte suspensions from the infected animals were negative for mycoplasma.

DISCUSSION

The partial or complete antibody suppression observed when *M. arthritidis* was injected concurrently with bacteriophage ϕ 5 is in agreement with unpublished observations of Kaklamanis *et al.* according to which *M. arthritidis* and *M. pneumoniae* interfere *in vivo* with the antibody response to DNP-BGG and SV 40 respectively.

Since no or little immunosuppression was observed when the micro-organism was injected 10 days after primary immunization, it might be suggested that *M. arthritidis* interferes with the induction and not the maintenance of immunity.

The partial or nearly complete inability of lymphocytes from infected animals to

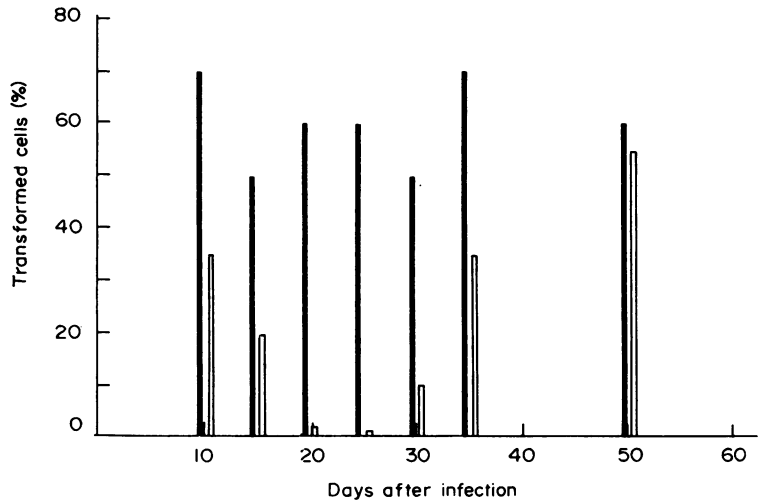

FIG. 3. Effect of PHA on lymph node lymphocytes. Solid columns indicate control (non-infected) animals; open columns indicate test (infected) animals (representative experiment).

respond to PHA parallels the results observed by several investigators that certain mycoplasma species inhibit blast transformation, when added *in vitro* (Copperman and Morton, 1966; Aula and Nichols, 1968; Barile and Levinthal, 1968; Simbercoff *et al.*, 1969).

The failure of lymphocytes of infected animals to transform *in vitro* and to produce antibody in response to bacteriophage ϕ 5 *in vivo* only when the infecting agent is given at the time of immunization could be explained by the effect of mycoplasma on a particular population of cells. This might represent the inability of a few specific infected cells to perform as immunocompetent cells. The fact that *M. arthritidis* was not isolated from the lymphocyte suspensions does not conflict with this suggestion as it is known that inhibition of blast transformation occurs as well *in vitro* in presence of dead mycoplasma (Copperman

and Morton, 1966) or mycoplasma extracts (Simbercoff *et al.*, 1969). Tissue extracts on the other hand contain a mycoplasmacidal factor which might have been responsible for the negative cultures (Kaklamanis, Thomas, Stavropoulos, Borman and Boshwitz, 1969).

If we accept that bacteriophage ϕ 5 is a thymus-dependent antigen (Marvanova and Hajek, 1969), both the inability of lymphocytes from infected animals to transform *in vitro* and to respond *in vivo* to the antigenic stimulus could be explained by the effect of mycoplasma on the T-lymphocytes. However, B-lymphocytes already committed to antibody production are not affected by the same micro-organism.

An alternative possibility is that mycoplasma interferes with the antigenic processing by macrophages which may be a necessary prerequisite for T-lymphocyte stimulation (Roitt, Greaves, Torrigiani, Brostoff and Playfair, 1969). Unpublished experiments by one of us in collaboration with Dr L. Thomas have shown that, after intravenous injection of *M. arthritidis* in rats, many peripheral leucocytes contain in their cytoplasm large pink inclusions, which were considered as phagocytosed mycoplasma.

While both the suppression of humoral immunity as well as the inhibition of mitogenic effect of PHA on lymphocytes from infected rats can satisfactorily be explained by the effect of *M. arthritidis* on a particular population of cells, the question of mechanism remains open.

There is a possibility that mycoplasma blocks specific receptors on the cell surface of antigen-sensitive cells. Thus, mycoplasma may act on the recognition site rather than on the cell function. This is supported both by the fact that mycoplasma is an extracellular organism (Jones and Hirsh, 1971) firmly attached on the cell membrane (Thomas and Kaklamanis, unpublished data) as well as by the observation that, in our system, immune lymphocytes continue to produce antibody in spite of the presence of mycoplasma infection.

The mechanism proposed by some investigators (Barile and Levinthal, 1968; Simbercoff *et al.*, 1969) of arginine depletion by *M. arthritidis* satisfactorily explains the *in vitro* inhibition of lymphoid blast transformation. The *in vivo* situation, however, is very complex and it is difficult for one to accept that mycoplasma is capable of depriving the animal nearly completely of arginine. Preliminary experiments have shown on the other hand that *M. pneumoniae*, a non-arginine utilizing mycoplasma, interferes to some extent with antibody production (Kaklamanis *et al.*, 1966). Thus, if arginine depletion by mycoplasma results in immunosuppression, this could not be the only mechanism *in vivo*.

Alternatively, immunosuppression might be due to competition between the mycoplasma and the bacteriophage antigens. Further experiments are in progress to elucidate the mechanism of mycoplasma-induced immunosuppression.

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